

# The effect of polyoxidonium on the phagocytic activity of human peripheral blood leukocytes

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## Abstract

The effect of polyoxidonium, a synthetic immunomodulator, which is a water soluble high-molecular compound, an N-oxidized polyethylene-piperazine derivative, on the functional activity of human peripheral blood phagocytic cells was studied *in vitro*. It was established that a one-hour incubation of leukocytes with polyoxidonium increased the ability of leukocytes to kill the ingested *Staphylococcus aureus* in a dose-dependent manner. This increase is observed with leukocytes obtained both from healthy persons and from patients with chronic granulomatous disease. The study of spontaneous and stimulated chemiluminescence of phagocytes showed a significant decrease in the quantity of chemiluminescent impulses in extracellular space in the presence of polyoxidonium both in luminol- and lucigenin-dependent chemiluminescence. Polyoxidonium proved to have antioxidant activity at all doses tested (100, 250, and 500 µg/ml). The analysis of intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) level by means of dichlorofluorescein, a fluorescent indicator, showed that incubation with polyoxidonium resulted in a higher luminescence intensity of dichlorofluorescein, thus indicating an increase in the intracellular H<sub>2</sub>O<sub>2</sub> level. This increase is not so considerable as it is in case with phorbol myristate acetate stimulation. However with polyoxidonium used at a dose of 500 µg/ml, the difference with the control is significant both for neutrophils and monocytes. Polyoxidonium can be used as adjuvant in combined treatment of acute and chronic infections of any etiology, in the treatment of CGD and secondary immunodeficiencies simultaneously with basic drugs.

**Key words:** immunomodulator, polyoxidonium, phagocyte, killing of bacteria, hydrogen peroxide, chemiluminescence

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## Introduction

The capacity of phagocytes to inactivate the ingested microbes is one of the crucial moments in the functioning of phagocytic cells, it is caused by oxygen-dependent and oxygen-independent factors. The clinical value of oxygen-dependent factors can be well demonstrated by the example of chronic granulomatous disease (CGD). Genetic deficiency of leukocytic NADPH-oxidase results in a practically absolute absence of reactive oxidant species thus leading to incomplete phagocytosis and severe clinical symptoms. Before the era of antibiotics, it caused an early lethal outcome [1, 2]. Nitric oxide (NO), another molecule which is highly toxic for a foreign microorganism, is

generated in a reaction catalyzed by inducible NO synthase (iNOS). In general, the enzyme activity of iNOS is revealed in macrophages, but there is evidence on mRNA iNOS expression by neutrophils obtained from septic patients and activated donor neutrophils [3, 4].

Management and prevention of diseases associated with disorders of bactericidal activity of leukocytes is based first of all on the use of antibiotics. Nowadays, however, an increase in the cases of chronic infectious inflammatory diseases may be observed which are characterized by a sluggish, recurrent course, resistance to adequate therapy and require additional immunostimulation. In patients with CGD, only bone marrow transplantation-with its own

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limitations due to HLA histocompatibility-can provide full recovery. A wide variety of CGD variants with a multiplicity of unique mutations causing a dysfunction of one enzyme (e.g., in 460 patients with X-linked CGD, X-CGD, about 290 various mutations in this chromosome were revealed [5]), involves various therapeutic approaches. For example, there are several reports on the improvement of clinical symptoms in some patients with CGD following the treatment with interferon  $\gamma$  (IFN $\gamma$ ) [1, 6, 7].

Polyoxidonium is characterized with immunomodulating activity targeted at the phagocytic link [8]. Polyoxidonium is a novel synthetic immunomodulator, a water-soluble high-molecular compound. By its chemical structure, it is a copolymer of N-oxidized 1,4-ethylenepiperazine and (N-carboxyethyl)-1,4-ethylenepiperazine bromide with a molecular weight of 100 kDa (Immafarma, National Research Center "Institute of Immunology", Moscow, Russia).

The aim of our work was to study the direct action of polyoxidonium on the most important functions of phagocytes, such as their ability to generate reactive oxidant species in the result of a respiratory burst and to kill foreign microorganisms ingested.

## Materials and methods

Leukocyte suspension was obtained from venous heparinized blood after spontaneous sedimentation of erythrocytes in a 1% gelatine solution. After the cells were twice washed in a phosphate buffered saline (PBS) with pH 7.4 (Sigma), they were resuspended in PBS and concentration of segmental neutrophils was brought up to 2 millions/ml in this cell suspension.

The polyoxidonium preparation (National Research Center "Institute of Immunology", Immafarma, Moscow, Russia) was used at the following doses: 100, 250, and 500  $\mu\text{g/ml}$ .

Chemiluminescence (CL) intensity was measured by means of 1251 Luminometer device (LKB). 300  $\mu\text{l}$  PBS were added to plastic test-tubes together with polyoxidonium at various doses (instead of polyoxidonium in control tubes, 300  $\mu\text{l}$  PBS were added). 100  $\mu\text{l}$  luminol solution (Sigma), 0.1 mg/ml, and 100  $\mu\text{l}$  cell suspension. After the peak value of spontaneous CL was measured, 10  $\mu\text{l}$  zymosan, 20 mg/ml (Sigma) – opsonized with pooled serum from 20 healthy donors-was added and the value of stimulated CL was measured (mV/min) [9].

Intracellular production of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was measured using dichlorofluorescein diacetate (DCF-DA) (Sigma) [10-12]. The reaction was performed in 96-well round-bottom culture dishes. Leukocytes were incubated at 37°C for 20 min with 5  $\mu\text{M}$  DCF-DA over 5 mM sodium azide. Then, equivalent doses of PBS with various doses of polyoxidonium were added, in control samples only PBS was added. After 60 min incubation with polyoxidonium at 37°C, the cells were centrifuged at 200 g for 1 min, erythrocytes were removed with lysing solution (200  $\mu\text{l/well}$ ), leukocytes were once

washed and re-suspended in 400  $\mu\text{l}$  PBS for further analysis by flow cytometer. As a criterion of system functioning, classic stimulator of phagocytes, phorbol myristate acetate (PMA; Sigma), was used. It was added at a dose of 100 ng/ml 30 min prior to incubation termination. The samples were analyzed by flow cytometer FACSCalibur (Becton Dickinson) with argon laser (488 nm) and the CELLQuest software. The intensity of fluorescence of granulocytes in the green spectral region was measured (GeoMean).

Intracellular killing of bacteria was evaluated by using fluorescein isothiocyanate (FITC) conjugated *Staphylococcus aureus* (*S. aureus*) [13]. The reaction was performed in 96-well round-bottom culture dishes (200  $\mu\text{l/well}$ ). 90  $\mu\text{l}$  leukocyte suspension (2 millions/ml), 90  $\mu\text{l}$  *S. aureus* (10 millions/ml), and 20  $\mu\text{l}$  pooled donor serum were incubated for 20 min at 37°C. To remove unbound bacteria, leukocytes were centrifuged at 200 g for 1 min at +4°C and twice washed with PBS. After it, leukocytes were re-suspended in 200  $\mu\text{l}$  PBS with polyoxidonium at various doses (only PBS was added to the control well) and incubated at 37°C for 60 min. Leukocytes were precipitated and resuspended in 200  $\mu\text{l}$  0.2% saponin solution (Sigma) in 10 mM carbonate-bicarbonate buffer (pH 9.5). The released bacteria were precipitated at 1,000 g for 10 min and resuspended in 200  $\mu\text{l}$  PBS with 2.5  $\mu\text{g/ml}$  propidium iodide (PI) (Sigma). After 10 min, the samples were analyzed by flow cytometer. The percentage of double positive bacteria (FITC\*PI\*) among FITC-labeled bacteria (FITC\*) was determined.

## Results

A characteristic functional feature of phagocytes is their capacity to generate reactive oxidant species in the course of respiratory burst. The effect of polyoxidonium on the levels of luminol- and lucigenin-dependent CL of phagocytes obtained from peripheral blood is summarized in table 1. Generation of reactive oxidant species into environment-that could be registered with luminophores (luminol emits light quantum after its reaction with  $\text{H}_2\text{O}_2$ , while lucigenin fluorescence is registered after its reaction with superoxide anion)-decreases greatly in the presence of polyoxidonium. A statistically significant decrease both in spontaneous and opsonized zymosan-stimulated CL signal was observed at all doses of polyoxidonium tested.

The effect of polyoxidonium on the intracellular level of  $\text{H}_2\text{O}_2$  was studied by means of DCF test. DCF-DA whose fluorescent properties are initially blocked penetrates cell cytoplasm and loses acetate groups after the treatment with intracellular esterase. The resultant DCF is a polar compound that cannot diffuse back and is highly sensitive to  $\text{H}_2\text{O}_2$ . Its reaction exactly with this active oxygen product restores fluorescent ability of DCF [10, 12]. When evaluating the effect of polyoxidonium on the intracellular  $\text{H}_2\text{O}_2$  level, we obtained results that are adverse to those of luminol- and lucigenin-dependent CL (Fig. 1). After one-hour incubation with

**Table 1.** The effect of polyoxidonium on chemiluminescence intensity of phagocytes obtained from donor peripheral blood

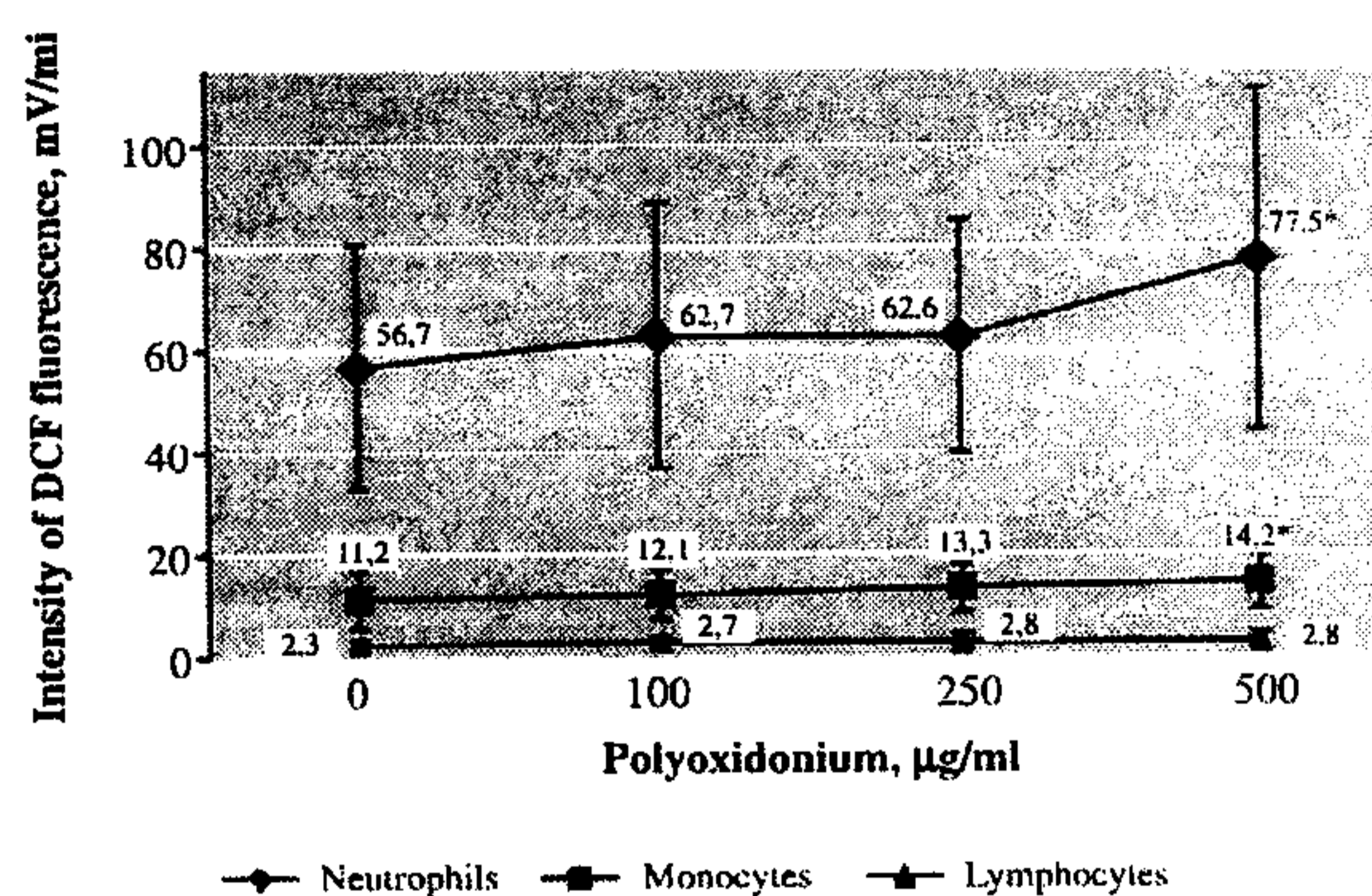
	Control	Dose of polyoxidonium, µg/ml		
		100	250	500
<b>Luminol-dependent chemiluminescence, mV/min</b>				
Spontaneous	9.1±5.9	5.0±3.5*	2.7±2.4**	1.5±1.0**
Stimulated	152.2±57.9	143.9±48.7	130.7±43.4*	109.2±30.1**
<b>Lucigenin-dependent chemiluminescence, mV/min</b>				
Spontaneous	17.4±9.4	4.5±5.5**	2.3±3.6**	1.2±1.5**
Stimulated	37.4±14.0	21.3±11.9*	17.4±10.2**	16.0±8.7**

Significance levels: \* $p < 0.05$ , \*\* $p < 0.01$  compared with the control (PBS)

polyoxidonium, the intensity of DCF fluorescence (Geo Mean) showed a trend towards an increase in the quantity of all kinds of leukocytes -lymphocytes including. It should be noted that compared with cell response to PMA (Geo Mean increases more than ten fold), the elevating effect of polyoxidonium is not so essential (Fig. 2). Nevertheless, at a dose of 500 µg/ml, polyoxidonium caused a statistically significant increase in the value of Geo Mean for neutrophils and monocytes. While the control Geo Mean level of neutrophils was 56.7±24.0, it reached 77.5±33.5 in the presence of polyoxidonium ( $p < 0.05$ ); the respective values for monocytes being 11.2±5.7 and 14.2±5.3 ( $p < 0.05$ ). On the average, the fluorescence intensity of neutrophils and monocytes increased by 40%. As to the intensity of lymphocyte fluorescence, it was also slightly elevated (by 20%).

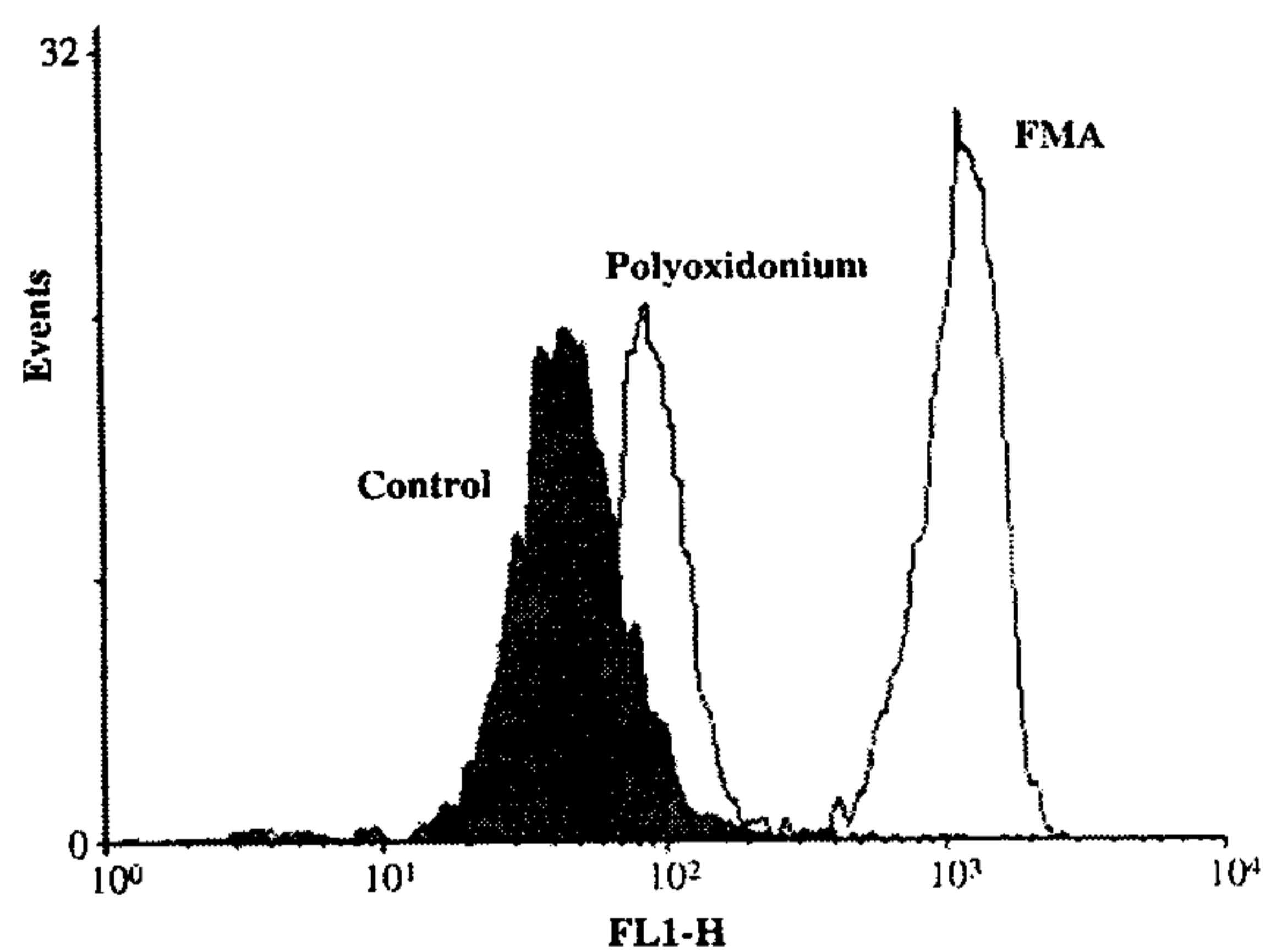
The main functional activity of phagocytes is their ability to kill the microbes ingested. The effect of polyoxidonium on bactericidal activity of phagocytes was studied by intracellular killing of FITC-labeled *S. aureus* with flow cytometry [13]. The preparation was added to cells that were already loaded with bacteria, that is, just for the killing period. Preliminary investigation of polyoxidonium ability to affect the ingesting activity did not reveal any effect in respect of both neutrophils and monocytes (data not presented). However, the percentage of intracellular killing of *S. aureus* increased significantly in the presence of polyoxidonium. A dose-dependent character of this elevating effect of polyoxidonium in donor cells is demonstrated with data presented in Table 2. At a dose of 250 µg/ml, polyoxidonium caused a 44% increase in bacteria killing level, while at a dose of 500 µg/ml this increase was 75%.

Bactericidal activity was also analyzed with phagocytes obtained from CGD patients. It was established that the ability of polyoxidonium to increase the efficiency of microbe killing revealed in the study of donor phagocytes becomes apparent with NADPH-oxidase deficient phagocytes from CGD patients. In this case, the killing-stimulating activity of polyoxidonium is not less at all. If taking into account the extremely low initial bactericidal level in CGD, these values even reach normal levels when



**Fig. 1.** The effect of Polyoxidonium on the level of intracellular hydrogen peroxide synthesis. The plot is based on the data of DCF test with donor leukocytes (n=15).

Significance level: \* $p < 0.05$  compared with the control



**Fig. 2.** Cytofluorograms of DCF-DA test. The effect of polyoxidonium on the intracellular level of hydrogen peroxide in donor peripheral blood neutrophils. Polyoxidonium, 500 µg/ml; phorbol myristate acetate (FMA), 100 ng/ml

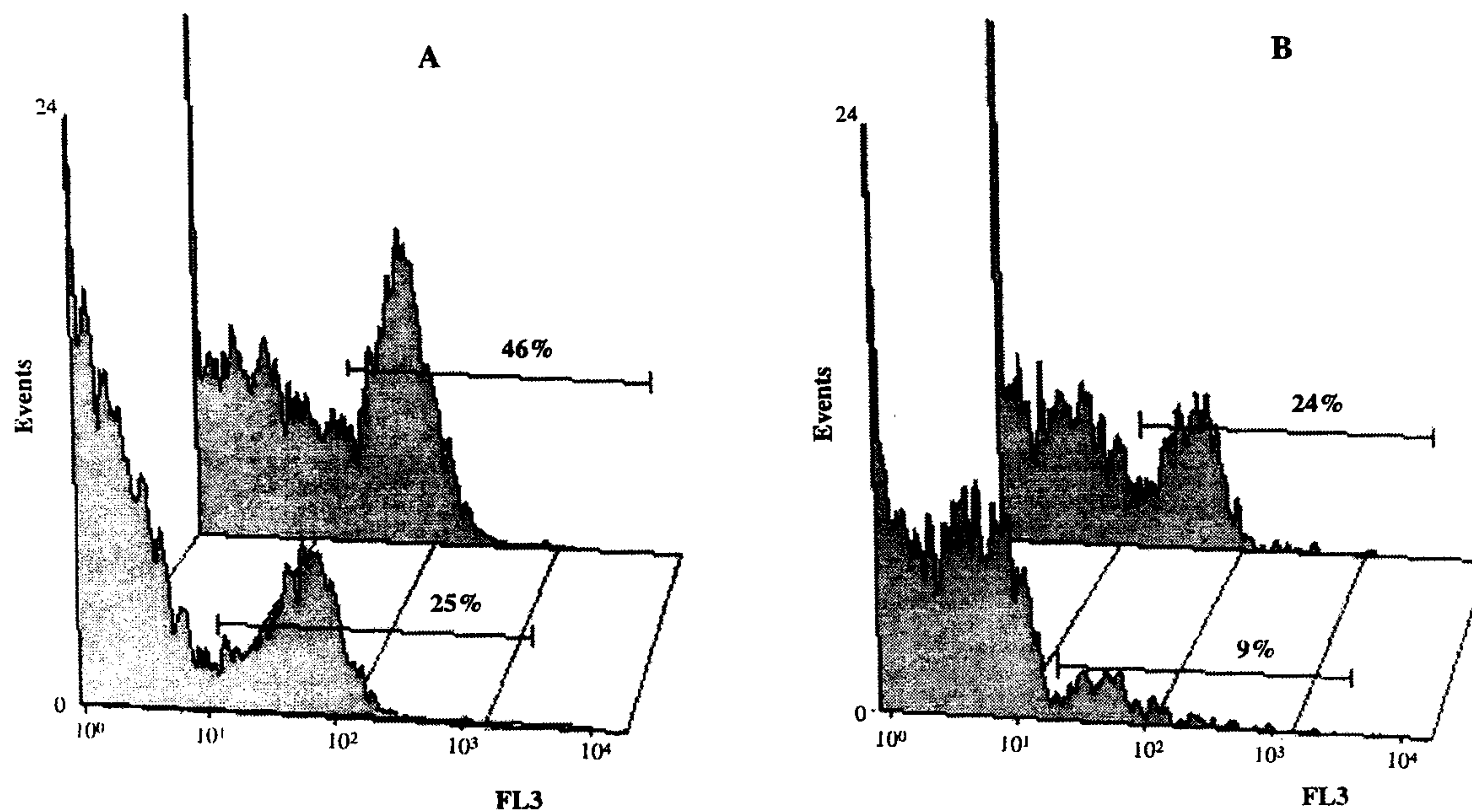
**Table 2.** The killing level (%) in leukocyte suspension obtained from donors and patients with chronic granulomatous disease in the presence of polyoxidonium

		Control	Dose of polyoxidonium, µg/ml		
			100	250	500
<b>Donors (n=11)</b>					
M±m		33.9±7.3	34.5±8.8	48.7±10.2**	59.3±6.8**
<b>Chronic granulomatous disease</b>					
Patients	1	7,5	12	18	30
	2	16	18	24	35
	3	13	20	22	35
	4	17	16	22	43
	5	9	12	24	41
M±m		12.5±4.2	15.6±3.6	22.0±2.4**	36.8±5.2**

Significance level: \* $p < 0.01$  compared with the control (PBS)

polyoxidonium is added at doses of 250 and 500 µg/ml (Table 2). Cytometric analysis of samples obtained from a CGD patient (histogram in figure 3) shows that the level of propidium iodide-positive bacteria (that is, killed bacteria) increases from the initial 9% to 24% under the effect of polyoxidonium.

To study the stimulating activity of polyoxidonium on intracellular killing of *S. aureus*, two inhibitors were used – sodium azide (blockage of respiratory chain enzymes) and methylmaleimide (bound to sulfhydryl groups in lysosome enzymes) -that block O<sub>2</sub>-dependent and O<sub>2</sub>-independent bactericidal mechanisms, respectively. The data obtained



**Fig. 3.** Cytofluorograms show intracellular killing of *St. aureus* by peripheral blood phagocytes obtained from a donor (A) and a patient with chronic granulomatous disease (B). In the foreground, there are control data (without polyoxidonium); in the background, data against polyoxidonium, 250 µg/ml. On the X scale, there is fluorescence intensity of FITC-labeled *St. aureus* on propidium iodide; on the Y scale, relative occurrence rate

in inhibitory analysis are presented in table 3. While in healthy persons the both inhibitors made a certain contribution to the activation blockage with polyoxidonium, in CGD persons, polyoxidonium increased the bacteria killing level in any inhibitor choice (sodium azide, methylmaleimide, sodium azide + methylmaleimide).

## Discussion

It is well known that the most important hallmark of the functional activity of phagocytes is the completeness of phagocytosis, that is, killing of bacteria ingested. The study of donors and four children with CGD revealed a unique ability of polyoxidonium to increase the percentage of intraphagocytic killing of *S. aureus* used in this reaction. Possible mechanisms of this stimulating effect of polyoxidonium were studied by means of inhibitory analysis. In healthy persons, sodium azide (blockage of O<sub>2</sub>-dependent bactericidity) and methylmaleimide (O<sub>2</sub>-independent mechanisms) reduced the level of intracellular killing of *S. aureus* stimulated with polyoxidonium. The killing level at that reached the control value, with the reaction performed without polyoxidonium and inhibitors (Table 3). In patients with CGD, however, polyoxidonium raised the percentage of bacteria killing with any inhibitor used (sodium azide, methylmaleimide, sodium azide + methylmaleimide). Thus, stimulating effect of polyoxidonium in CGD – least of all associated with activation of O<sub>2</sub>-dependent bactericidal mechanisms (the genetic defect rules it completely out [1, 14]) – is fulfilled through other mechanisms that are not blocked with methylmaleimid either. Stimulation of intracellular killing of *S. aureus* may be associated with activation of NO-dependent pathway of bacteria death. NO is of great value for killing of *Mycobacterium tuberculosis*, *Plasmodium falciparum* and other pathogenic

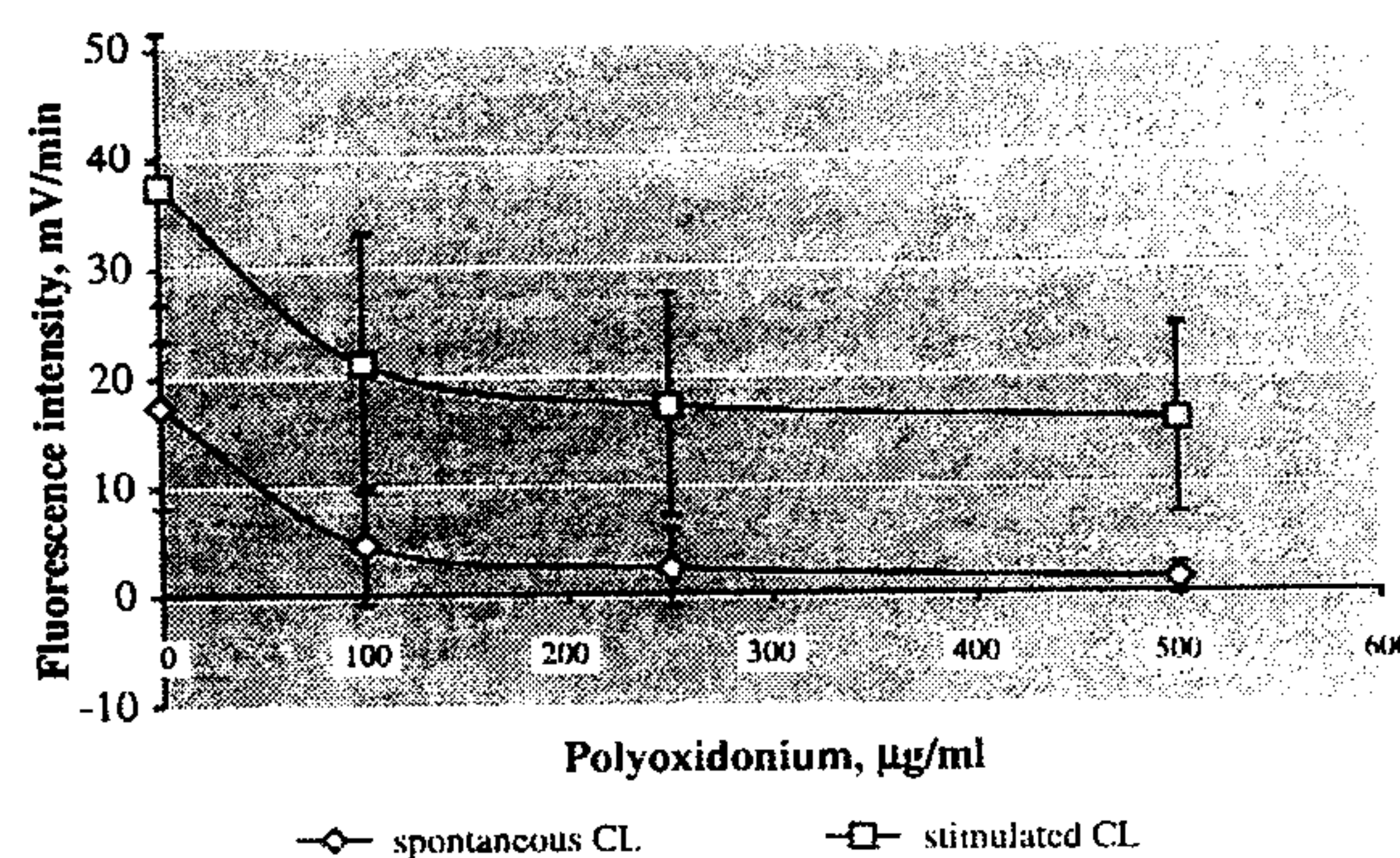


Fig. 4. The decrease in the intensity of spontaneous and stimulated lucigenin-dependent chemiluminescence of donor leukocytes (n=8) under the effect of various doses of polyoxidonium

microorganisms [15-17]. Expression of iNOS enzyme (NOS-2, another name) that is responsible for NO synthesis in phagocytes-macrophages, monocytes, and neutrophils, in particular-is of inducible character, as distinct from two other known constitutive enzyme isoforms (NOS-1, NOS-3). It is known that iNOS expression is induced by microbe products, inflammatory cytokines, and tumor cells [3, 4, 15-19].

Very interesting data were obtained in our study of polyoxidonium effect on the generation of reactive oxidant species registered with CL analysis. A significant lowering of chemiluminescent signal revealed, both luminol- and lucigenin-dependent (Table 1), may testify either to the ability of polyoxidonium to directly suppress the activity of leukocyte NADPH-oxidase or to a powerful antioxidant activity of polyoxidonium.

An argument to testify against the first possible mechanism is that the test procedure of the study of

Table 3. The effect of inhibitors (sodium azide, 0.1%; methylmaleimide, 1 mM) on the intracellular killing of *S. aureus* in blood samples obtained from donors and patients with chronic granulomatous disease against polyoxidonium, 500 µg/ml

	Without polyoxidonium				With polyoxidonium			
	PBS	Na azide	M-maleimide	Na azide + M-maleimide	PBS	Na azide	M-maleimide	Na azide + M-maleimide
<b>Donors (n=5)</b>								
M±m	33.0±6.0	20.1±2.7**	19.0±6.2*	16.7±5.0**	56.8±13.3	37.2±7.2*	40.0±14.1	35.0±9.9*
<b>Chronic granulomatous disease</b>								
1.	17	17	16	n.d.	43	32	38	n.d.
2.	16	17	12	11.5	35	28	32	32
3.	13	20	12,5	12	35	28	32	35
4.	9	8	9	8	41	35	38	46
M±m	13.8±3.6	15.5±5.2	12.4±2.9	10.5±2.2	38.5±4.1	30.8±3.4	35.0±3.5	37.7±7.4

Significance levels: \*p<0.05, \*\*p<0.01 compared with the control (PBS)

polyoxidonium effect on CL does not include preliminary incubation of cells with the preparation (see Materials and methods). Momentary inhibition of a whole enzyme complex – which is NADPH-oxidase – is hardly possible. Second, it could not escape to tell upon the intensity of intracellular killing of *S. aureus*. Analysis of the degree of chemiluminescent signal reduction, both in spontaneous and zymosan-stimulated probe, revealed that its level in the presence of polyoxidonium CL is lowered by the same degree in both probes if the same dose of polyoxidonium is used (Fig. 4). Hence, antioxidant “capacity” of polyoxidonium directly depends on its dose.

If chemiluminescent signals that are emitted by luminophores reflect the level of reactive oxidant species that are produced by cells into environment-lucigenin is oxidized and luminesces under the effect of superoxide anion, luminol-dependent CL is produced in myeloperoxidase system-to study the level of intracellular reactive oxidant species, various fluorochromes are used that are activated while cooperating with these radicals in cell cytosol directly. DCF-DA used in our study is an indicator of intracellular  $H_2O_2$ . By means of flow cytometry, it is possible to evaluate the intensity of DCF fluorescence (Geo Mean) and thus, assess the level of  $H_2O_2$  in every single cell.

It is generally accepted, that oxygen radicals,  $H_2O_2$  in particular, participate in the damaging of membranes and of the microbe cellular DNA. At the same time, they are toxic for ambient cells of the body. Our study of polyoxidonium effect on the Geo Mean value of DCF-DA-treated leukocytes revealed a dose-dependent increase of intracellular  $H_2O_2$ , however the increase of the Geo Mean value in response to polyoxidonium are non-comparable to the level of Geo Mean in the induction of respiratory burst.

Recent studies show that a slight elevation in the level of  $H_2O_2$  is crucial for activation of some signaling molecules [2, 20-24] and is of great biological value like the phenomenon of respiratory burst.  $H_2O_2$  was proved to participate in the process of activation of the important transcription factor NF- $\kappa$ B [21, 25, 26]. The number of genes transcriptionally regulated by NF- $\kappa$ B continues to expand, and includes genes that regulate inflammation and immune responses, viral replication, nitric oxide production, cell-cell interaction, apoptosis, proliferation, etc. [26].

Synthesis of macrophage vascular endothelial growth factor (VEGF) was shown to be induced by  $H_2O_2$  [27].  $H_2O_2$  also participates in the activation of phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1). PLC- $\gamma$ 1 also plays a key role in regulation of cell proliferation and differentiation through the induction of secondary messengers-diacylglycerol and inositol-3-phosphate that cause activation of protein kinase C (PKC) and mobilization of  $Ca^{2+}$  [24]. These studies were performed on macrophage culture U937. In the culture of human lung fibroblasts (HLF), it was demonstrated that stimulation of these cells with transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) provides a transitory elevation of the  $H_2O_2$  level that, in its

turn, induces an increase in the level of  $Ca^{2+}$  with further activation of mitogen-activated protein kinase (MAPK), AP-1 and results to interleukin (IL)-6 gene expression [2].

High levels of  $H_2O_2$  are known to activate stress-activated protein kinase (SAPK) in U937, one of cell death mediators [22]. At the same time, the authors have demonstrated this effect to be abolished completely if U937 was pretreated with low doses of  $H_2O_2$ .

Inactivation of glutathione peroxidase (GxP) leads to the accumulation of  $H_2O_2$  and the induction of signaling pathway via c-Jun NH2-terminal kinase (JNK) in rat aortic smooth muscle cells [23]. It is also stated that  $H_2O_2$  activates STAT3 and, to a less degree, STAT1 in fibroblastoid cells [20].

The elevated  $H_2O_2$  level in donor neutrophils and monocytes, observed in our experiments after a one-hour incubation with polyoxidonium, may point to its ability to affect the intracellular signaling mechanisms. This fact can explain our previous data on the polyoxidonium induction of IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  synthesis by donor peripheral blood monocytes. The level of this induction is the same as with the classic inductor of both cytokines mentioned, lipopolysaccharide (LPS) [8]. Further study of the mechanisms of action of polyoxidonium with the aim to identify the major signaling molecules, would reveal molecular-biological mechanisms of its immunomodulating activity.

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